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Abstract

Selenium-accumulating plants such as *Brassica juncea* (Indian mustard) concentrate the element in plant shoots and roots. Such behavior may provide a cost-effective technology to clean up contaminated soils and waters that pose major environmental and human health problems (phytoremediation). Such ability to transform selenium into bioactive compounds has important implications for human nutrition and health. Element selective characterization of *B. juncea* grown in the presence of inorganic selenium under hydroponic conditions provides valuable information to better understand selenium metabolism in plants. The present work determines both previously observed organoselenium species such as selenomethionine and Se-methylselenocysteine and for the first time detects the newly characterized S-(methylseleno)cysteine in plant shoots and roots when grown in the presence of selenate or selenite as the only selenium source. A key feature of this study is the complementary role of selenium and sulfur specific chromatographic detection by HPLC with interfaced inductively coupled plasma mass spectrometry (ICP-MS) detection and by derivatization GC with interfaced atomic spectral emission. HPLC–ICP-MS limits of detection for such species were in the range 5–50 ng Se mL^{-1} in the injected extracts. Speciation profiles are compared with those of selenium-enriched yeast by both HPLC–ICP-MS and GC–AED.

Keywords: Selenium; *Brassica juncea*; Yeast; Speciation

1. Introduction

Phytoremediation, the use of plants to accumulate and hence remove environmental pollutants, has been proposed as a cost-efficient technology for the elimination of toxic contaminants from the environment [1]. Certain plants remove different pollutants from contaminated water or soil by absorption through the roots followed by transportation to stems, shoots, and leaves. The plant can then be harvested and removed from the site. Plants may transform some toxic contaminants to environmentally harmless volatile species, which may be dispersed away from polluted areas [2]. Such detoxification processes have been used for the elimination of pesticides or polycyclic aromatic hydrocarbons (PAHs) from soils, and recently, successful results have been obtained for removing selenium from soils [3,4] and hydroponic media [5–8].

Selenium is an essential nutrient for animals and has cancer chemopreventive properties for humans [9]. Dietary deficiencies exist in many countries because low soil results in low selenium in the food chain [10]. Inverse associations exist between nutritional selenium and cancer risks, cardiovascular disease, and immune system functions [11–13]. Selenium fertilization of vegetable crops has been used to increase dietary selenium levels in humans and other animals [14]. Growing plants enriched with selenium could be an effective way to reduce dietary deficiencies and increase health benefits [15,16]. Selenium has not been classified as a plant essential element, but its role as a beneficial element in plants capable of accumulating large amounts of selenium has been considered [17]. Uptake and accumulation of selenium by plants is deter-

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mined by selenium form and concentration, the identity and presence of competing ions, and the affinity of a species to absorb and metabolize selenium [5]. Actively growing tissues usually contain the highest amounts of selenium. Many plant species accumulate higher amounts of selenium in shoot or leaf tissues than root tissues, but there are exceptions noted in the literature [5,18].

Selenium-enriched media affected seed germination in a number of species. Seed germination was reduced if wheat (*Triticum aestivum* L.) was grown in soils with >16.0 mg Se kg^{-1} [19]. In another study suppressions in alfalfa (*Medicago sativa* L.) seedling fresh weights were reported in response to >10.0 mg Se L^{-1} in solution cultures [20]. Turnip (*Brassica campestris* L., Rapifera Group) seed germination was >98% when seeds were incubated in <484 mg Na_2SeO_3 L^{-1} (2.80 mM), but fell to 51% if Na_2SeO_3 concentration was increased to 4.84 g Na_2SeO_3 L^{-1} (28.0 mM) [21]. Interestingly, several studies report that seed germination was enhanced in response to <1.0 mg Se L^{-1} in nutrient solutions [22,23]. Activity of β-galactosidase, an enzyme important in the hydrolysis of complex carbohydrates during seed germination, in *Trigonella foenum-graecum* was enhanced by 40% when exposed to 0.5 mg Na_2SeO_3 L^{-1} seed treatment, but decreased 60–65% if Na_2SeO_3 seed treatment was increased to 1 mg L^{-1} [24]. These studies established toxicological tolerances to external Se concentrations for seed germination.

Although most fruits and vegetables contain <0.01 μg Se g^{-1}, some species are Se-enriched when grown in a high selenium environment. These include the 'brassicas', which also synthesize organic selenium compounds of potential therapeutic value. A member of the Brassicaceae family, Indian mustard (*Brassica juncea*) has potential for selenium phytoremediation because of its fast growing cycle and high biomass. In this paper the selenium species identified in hydroponically grown *B. juncea* shoots and roots are presented. Speciation profiles are also compared, qualitatively and quantitatively, with those in freshly prepared and stored high selenium yeast, by both HPLC–ICP-MS and GC–AED. The goal of this study is to identify and quantify the different selenium species produced by *B. juncea* in order to help elucidate the selenium metabolic pathway in selenium accumulators such as *B. juncea*.

2. Experimental

2.1. Instrumentation

An ELAN 5000 inductively plasma mass spectrometer (Perkin-Elmer Sciex, Norwalk, CT, USA) was used as a HPLC detector and for total selenium determination. Samples were introduced into the ELAN 5000 with a cross flow nebulizer, and a Scott type double pass spray chamber. The inductively coupled plasma mass spectrometry (ICP-MS) conditions were as follows: power 1000 W, plasma argon flow 15 L min^{-1}, auxiliary flow 0.8 L min^{-1}, nebulizer flow 0.8 L min^{-1}, isotope monitored ^{82}Se, scanning mode peak hop, dwell time 100 ms. The HPLC system consisted of a liquid chromatograph (Applied Bio Systems/model 400 solvent delivery system, San Jose, CA, USA). Injections were made using a Model 7725 injection valve (Rheodyne, Cotati, CA, USA). The HPLC column was connected to the nebulizer of the ICP-MS instrument by polyether ether ketone (PEEK) tubing (20 cm × 0.25 mm).

The chromatographic columns evaluated (Waters Corporation, Milford, MA, USA) were (a) a 5 μm XTerra RP-C18 (4.6 mm × 15 cm) column, a hybrid particle that has a polar modifier group between the C18 group and the silica base, (b) a 3.5 μm XTerra MS-C18 (4.6 mm × 15 cm) column, (c) a 5 μm Symmetry Shield RP-C18 (3.9 mm × 15 cm) column and (d) a 5 μm Symmetry Shield RP-C8 (3.9 mm × 15 cm) column. Mobile phase was 0.1% HFBA, 1% methanol, 99% water, flow rate was 1 mL min^{-1} and injection volume was 20 μL.

Data were collected using Perkin-Elmer ELAN software in the graphics mode, processed with PeakFit™ (version 4) software and plotted with Microsoft Excel software.

A Hewlett Packard HP 5921A atomic emission detector interfaced with a HP 5890 II gas chromatograph was used for GC–AED studies. The chromatographic separation was achieved using a 30 m fused capillary column SE-30 with 0.32 mm internal diameter and 0.25 μm film thickness. The helium carrier flow rate was 1 mL min^{-1} and the injector split ratio was 30:1. The helium plasma gas flow was kept at 180 mL min^{-1}. Hydrogen was used as reagent gas, with detection at 181 nm (S) and 196 nm (Se). The oven was held at 60 °C for the first 10 min and then heated to 300 °C at a rate of 5 °C min^{-1} and held there for 2 min. The run time was 60 min.

For total selenium determination a Perkin-Elmer 4100ZL electrothermal atomic absorption spectrometer equipped with longitudinal Zeeman-background correction and a transversely heated graphite atomizer (THGA) tube, equipped with an integral L'vov platform (Perkin-Elmer part number BO504053) was also used. A Perkin-Elmer AS–70 autosampler injected the standards and samples into the graphite furnace. The selenium electrodeless discharge lamp (EDL) from Perkin-Elmer operated at 260 mA with a slit width of 2 nm, a wavelength of 196 nm and powered by a Perkin-Elmer system 2 EDL. The inert carrier gas was argon (Merriam–Graves, high purity), the flow rate being 250 mL min^{-1} during all stages of the furnace program, except during the atomization step when the gas flow was stopped. Perkin-Elmer recommended conditions were used for total selenium determination.

For the closed vessel microwave-assisted acid digestion of the samples a MDS 2100 microwave oven (CEM, NC, USA) with PTFE vessels was used. LABQUAKE® shakers and rotators (Barnstead/Thermolyne Corporation, Dubuque, IA, USA) and a centrifuge (Fisher Scientific, Fair Lawn, NJ, USA) were also used. The glassware used was nitric acid washed before and after use, and rinsed with ultra pure water

(18 MΩ Barnstead L-pure, Boston, MA, USA). Deionized water was used throughout the experiments.

2.2. Chemicals and samples

Barnstead E-pure 18 MΩ water (Boston, MA, USA), methanol (HPLC grade), HFBA (Aldrich, Milwaukee, WI, USA) was used. Sodium selenate, sodium selenite, selenocystine, selenoethionine, methionine, ethionine, and Protease XIV were obtained from Sigma Chemical Company (St. Louis, MO, USA). The selenium and sulfur containing species S-(methyseleno)cysteine was synthesized in the laboratory of Professor Eric Block (State University of New York, Albany, NY, USA). Dilute HCl solutions of 2 mg mL^{-1} Se-methylselenocysteine, 5.2 mM Se-allylselenocysteine and 13.1 mM Se-propylselenocysteine were obtained from Professor Howard Ganther (University of Wisconsin, Madison, WI, USA). Selenized yeast was obtained from LeSaffre yeast Corporation (Red Star nutritional yeast). Ethylchloroformate (ECF) (Sigma-Aldrich, St. Louis, MO, USA), ethanol and pyridine (Fisher Scientific) were used for GC derivatization. Hexane and chloroform (Fisher Scientific) were used as extraction solvents. Selenium, rhodium and lead standard solutions (1000 μg mL^{-1}) were obtained from Spex (Spex Industries Inc., Edison, NJ, USA). Palladium (10,000 mg L^{-1} as nitrate) and magnesium nitrate (10,000 mg L^{-1}) solutions for ET-AAS analysis were obtained from Perkin-Elmer. For the extractions hydrochloric acid and for microwave digestions nitric acid and 30% (v/v) hydrogen peroxide (Fisher Scientific) were used.

A stock solution of the selenium-containing standards was prepared in 0.2 M HCl and stored in a refrigerator below 4 °C.

For the Hoagland nutrient solutions 40 mL of 0.5 M potassium dihydrogen phosphate, 200 mL of 0.5 M potassium nitrate, 80 mL of 0.5 M magnesium sulfate, 200 mL of 0.5 M calcium nitrate, 20 mL of 0.01 M iron chelate—NaFeEDTA and 20 mL of micronutrient solutions were used. All reagents were of analytical reagent grade (Fisher Scientific).

2.3. Plant growth and sample preparation

B. juncea seeds (accession number 426314) were obtained from the USDA-ARS in Ames, IA. The seeds were germinated in water-moisturized rock wool for a period of 7 days, and subsequently the seedlings were transferred into 1.5 L hydroponic media. Seedlings were supported by moist rock wool placed on the lids, where the roots were in contact with the nutrient solution. Solutions were continuously aerated and filled with Hoagland's nutrient solutions weekly [25]. The molar composition was as follows: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4$ and 0.01 mM NaFeEDTA in 20 L solution. Twenty-four plants were grown, 10 plants for selenate (as Na_2SeO_4) treatments, 10 for selenite (as Na_2SeO_3) treatments and 4 were used as controls (blank with no selenium). Plants were cultivated in a greenhouse at controlled temperature of 25 ± 2 °C and with a 12-h daylight period. After 30 days, the hydroponic solutions were spiked with aqueous sodium selenate or sodium selenite to give a final solution concentration of 5 μg Se mL^{-1}. A previous study showed suppressions in plant biomass production in nutrient solutions amended with >9 mg Na_2SeO_4 L^{-1} [26]. Plant heights were measured regularly to monitor the growth after the selenium treatments. Plants were harvested and separated into shoots and roots and carefully washed with distilled water. The different plant parts were weighed (wet mass), placed in paper bags and dried in an oven at 60 °C for 48 h. The dry plant parts were weighed (dry mass) and ground with mortar and pestle. The finely ground material was passed through a 250 μm sieve before further analysis.

2.4. Total selenium determination

For the determination of total selenium by ICP-MS and ET-AAS, approximately 0.2 g of dry plant material (shoots and roots) were microwave digested using 2 mL of HNO_3 (70%) and 1 mL of H_2O_2 (30%), employing the following four step digestion program, based on 1000 W full power. Step 1: 2 min at 25% power, 20 psi and 120 °C, step 2: 5 min at 66% power, 40 psi and 140 °C, step 3: 5 min at 66% power, 85 psi and 160 °C, step 4: 5 min at 66% power, 90 and 180 °C. The digested clear solutions were transferred and made up to volume with de-ionized water in 10 mL calibrated flasks. The total selenium concentrations in plant shoots and roots were determined by ICP-MS and ET-AAS using the standard addition method.

3. Extraction procedures

3.1. Enzymatic hydrolysis

Approximately 0.5 g of the powdered sample and 0.02 g of the enzyme (Protease XIV) was placed in a 15 mL centrifuge tube. After addition of 5 mL of distilled deionized water, the samples were shaken for 24 h at room temperature using LABQUAKE® Shakers and Rotators (Barnstead/Thermolyne Corporation, Dubuque, IA, USA).

After extraction the samples were centrifuged at 3000 × *g* for 20 min (Fisher Scientific). Then the supernatant was filtered through a 0.45 μm polypropylene filter (Whatmann®). The filtered supernatant was once again filtered through a 10,000 Da molecular weight cut-off 'Ultrafree-4' centrifugal filter and tube (Millipore Corporation, Bedford, MA, USA) by centrifugation for 50 min. The clear filtrate was mixed with HFBA (900 μL of the extract and 100 μL of HFBA) before HPLC–ICP-MS analysis.

3.2. HCl acid extraction

Approximately 0.5 g of the powdered plant material was mixed with 5 mL of the HCl acid extraction solution. Three different HCl concentrations (0.5, 1 and 2 M) were examined. After extraction the samples were centrifuged at 3000 × *g*

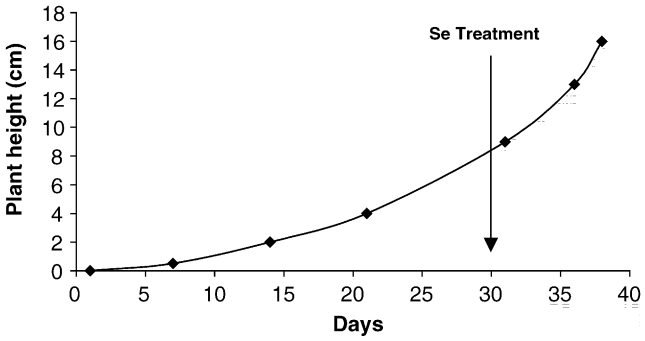

Fig. 1. Plant growth of *B. juncea* during 40-day growth period. Selenium treatment with selenite (Se(IV)) and selenate (Se(VI)) after 30 days.

for 20 min, and then the supernatant was filtered through a 0.45 μm polypropylene filter. The clear filtrate was used for HPLC–ICP-MS analysis.

3.3. Derivatization of selenoamino acids for GC–AED

Selenoamino acid standards were derivatized with ethylchloroformate using the method developed by Husek [27]. Stock solutions of the amino acids were prepared by weighing 10 mg samples and dissolving in 1 mL of 0.1 M HCl to make 10,000 ppm standard solutions. Then 1000 ppm derivatized standard solutions were prepared as follows: 100 μL of the 10,000 ppm selenoamino acid stock solution was treated with 1 mL of water–ethanol–pyridine mixture (60:32:8), 50 μL of ECF was added and the mixture shaken until gas evolution ceased. Then 1 mL of chloroform (containing 1% ECF) was added and the derivatives were extracted into the organic phase, which was evaporated to dryness. The residue was redissolved in 1 mL of hexane and 1 μL was injected.

A 2-mL portion of the aqueous (non-acidified) extract prepared for HPLC analysis was also taken for examination by GC–AED. A water:ethanol:pyridine mixture (60:32:8 by volume, 15 mL) was added followed by ethylchloroformate (ECF, 1 mL), the sample was shaken and any evolved gas vented. Ethylated derivatives were extracted into 3-mL chloroform (containing 1% ECF) by shaking for 30 min. The chloroform layer was separated, solvent was removed with a flow of nitrogen and the residue was redissolved in 100 μL chloroform or hexane. One microliter was injected.

4. Results and discussion

4.1. Selenium accumulation

The *B. juncea* plants used in this study were grown hydroponically for 40 days with selenium nutrient supplementation in the form of selenite (Se(IV)) or selenate (Se(VI)). The plants were grown for 30 days before adding the selenium, the above ground plant heights being recorded periodically; the selenium added to the growing medium did not appear to inhibit plant growth (Fig. 1). Visual symptoms of selenium toxicity did not appear in the plants when grown in the presence of selenium and no differences in biomass production were noticed. Selenium concentrations in different plant shoots and roots were determined to assess the variability of selenium accumulation among the different *B. juncea* plants for each treatment.

4.2. Determination of total selenium by GF-AAS and ICP-MS

For the determination of total selenium, three plants from the selenate treatment and three plants from the selenite treatment were randomly selected. While these might not be representative of the full growth batch, the data obtained indicates a probable selenium content range. The plant shoots and roots were microwave acid digested and the selenium content was determined by ICP-MS and electrothermal atomic absorption spectrometry (ET-AAS) for comparison purposes. Table 1 shows that higher selenium concentrations were found in selenate treated plant shoots (1008–1391 μg Se g^{-1}) as compared to selenite treated plant shoots (61–121 μg Se g^{-1}). However, the selenium concentration in the selenate treated

Table 1
Comparison of the results for the determination of selenium in plants

Selenium	Shoot concentration (μg g^{-1})[a]		Root concentration (μg g^{-1})[a]	
	ICP-MS[b]	ET-AAS[b]	ICP-MS[b]	ET-AAS[b]
Selenite (Se(IV))				
Plant # 1	85 ± 8	92 ± 18	326 ± 65	340 ± 93
Plant # 2	61 ± 4	58 ± 2	204 ± 36	200 ± 11
Plant # 3	121 ± 10	109 ± 23	406[c]	424[c]
Selenate (Se(VI))				
Plant # 1	1013 ± 24	1003 ± 34	110 ± 13	111 ± 23
Plant # 2	1008 ± 48	1056 ± 59	76 ± 9	65 ± 4
Plant # 3	1391 ± 92	1331 ± 84	146 ± 7	134 ± 12

[a] Mean ± 95% confidence level.
[b] Treatments.
[c] Only one replicate measured (low sample mass).

plant roots were lower (76–146 μg Se g^{-1}) than in the selenite treated plant roots (204–406 μg Se g^{-1}). These findings are in agreement with other reports [17]. The translocation of selenium from root to shoot is dependent on the form of selenium supplied, selenate being transported to the plant shoots much more easily than selenite. In the selenite treatments most of the selenium remained in the plant roots and only a small fraction was found in the plant shoots. It can be concluded that enrichment procedures in *B. juncea* with selenate proved to be more effective in terms of selenium accumulation than with selenite.

4.3. Extraction of selenium species in plants

The common procedures used for the speciation of selenium in yeast, plants and vegetables have been hot water extraction, enzymatic hydrolysis, buffers, water–methanol and HCl extraction [6,7,28–30]. In this study, protease XIV enzymatic extraction was used to cleave peptide bonds in proteins in order to characterize low molecular weight selenium compounds in the plants. Other preliminary experiments suggested low extraction efficiencies (ca. 37% based on microwave digestion for total Se) for the HCl extractions as compared with higher extraction efficiencies for the enzymatic extractions.

4.4. Chromatographic conditions for selenium speciation by HPLC–ICP-MS

The reversed-phase perfluorinated ion-pairing HPLC method is a robust method for speciation of selenium compounds in natural products. In previous studies this method was developed and extensively used for selenium speciation in yeast and more recently used for speciation in plants [31,32]. Different perfluorinated ion-pairing reagent mobile phases such as trifluoroacetic acid (TFA), pentafluoropropanoic acid (PFPA), heptafluorobutanoic acid (HFBA) and nonafluoropentanoic acid (NFPA) have been examined for the separation of inorganic selenium and seleno-amino acids. The concentration of the ion-pairing reagent, the pH of the mobile phase and the pH of the sample extracts were optimized to achieve adequate separation of the selenium species. In general, when TFA is used in the mobile phase, the early eluting peaks are poorly resolved but the peak resolution of the later part of the chromatogram is very good. The separation power at the beginning of the chromatogram increased with greater chain-length of the ion-pairing agent. The separation provided by HFBA and NFPA gave generally satisfactory results throughout the chromatogram but resulted in lengthy elution times. An XTerra reversed phase C18 column that has a bonded hybrid stationary phase was used in these experiments. The XTerra column differs from typical C18-silica columns in the reduction of accessible silanol groups, one-third of the stationary phase surface sites having C18 groups, one-third methyl groups, and the remaining one-third has sterically hindered residual silanols. The XTerra column can be used over a wide pH range (2–12) and a wide temperature range (20–60 °C) and provides decreased overall retention times over the widely used 'Symmetry Shield' column with 0.1% HFBA pairing agent. The retention time for the selenomethionine peak in the standard mixture was decreased from 12.9 min with the Symmetry Shield to 6.1 min with the XTerra column for otherwise identical operating conditions. A typical HPLC–ICP-MS chromatogram for four selenium species is shown in Fig. 2. Under these chromatographic conditions S-(methylseleno)cysteine elutes at 5.3 min, selenomethionine Se-oxide hydrate elutes at 3.1 min and Se-methylselenocysteine elutes at 3.5 min.

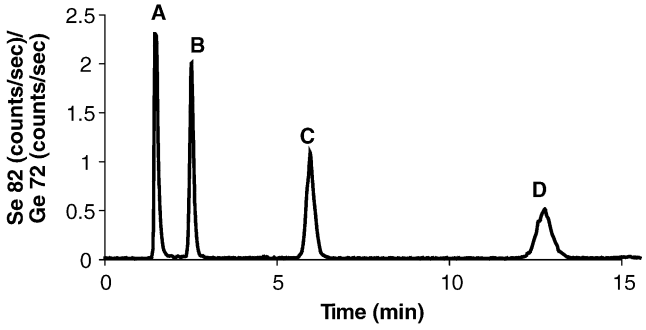

Fig. 2. HPLC–ICP-MS chromatogram of mixed selenium standard solution using 0.1% HFBA ion pairing agent, XTerra RP-C18 column: (A) selenite; (B) selenocystine; (C) selenomethionine; (D) selenoethionine.

The pH of 0.1% HFBA mobile phase is 2.40, which is in the pK_a region for the carboxylic groups of the amino acids. At this pH, the amino acid carboxylic group will be deprotonated and the amino group protonated. Change in pH of the mobile phase modifies the on-column equilibrium conditions. When the ion-pairing agent concentration was increased (0.25% HFBA) or the pH was adjusted by adding concentrated HCl to the mobile phase, the retention times increased and some peaks co-eluted (data not shown). Therefore, it is important to select the mobile phase ion pairing agent concentration and control pH carefully.

4.5. HPLC column evaluation

The chromatographic parameters for the HPLC–ICP-MS separation of the selenium standard mixture were examined using different reversed phase columns in terms of capacity factors, resolution, the number of theoretical plates and peak symmetry. These values are tabulated in Table 2. Capacity factors were calculated for a void volume of 1 mL, ideally capacity factors between 1 and 10 being desirable. The peak asymmetry values (A_s) were calculated using PeakFit software, where $A_s = b/a$, for which 'b' is the width to the left of the peak and 'a' is the width to the right of the peak, each measured at 10% peak height. The XTerra RP-C18 and Symmetry Shield RP-C18 columns show good separation for the selenium species examined.

Table 2
Chromatographic parameters for selenium standards using different LC columns

Column	Selenium species	t_R[a]	k'[b]	R[c]	N[d]	Asymmetry[e]
5 μm XTerra RP-C18 (4.6 mm × 15 cm)	Selenite	1.5	0.5		698	1.62
	Selenocystine	2.51	1.51	4.1	2239	1.07
	Selenomethionine	5.96	4.96	8.1	2479	1.63
	Selenoethionine	12.73	11.73	8.9	4424	1.2
3.5 μm XTerra MS-C18 (4.6 mm × 15 cm)	Selenite	1.5	0.5		786	2.6
	Selenocysteine	5.1	4.1	5.7	1794	24.9
	Selenomethionine	38.7	37.7	22.7	8510	1.07
5 μm Symmetry Shield RP-C18 (3.9 mm × 15 cm)	Selenite	1.4	0.4		308	1.39
	Selenocystine	5.3	4.3	7.9	1719	1.07
	Selenomethionine	27.3	26.3	19.4	6015	1.15
5 μm Symmetry Shield RP-C8 (3.9 mm × 15 cm)	Selenite	1.1	0.1		721	1.56
	Selenocystine	2.9	1.9	2.9	1154	1.43
	Selenomethionine	13.2	12.2	8.7	7520	1.08
	Selenoethionine	38.7	37.7	9.2	9518	1.09

[a] Retention time.
[b] Capacity factor based on 1 mL void volume.
[c] Resolution.
[d] Theoretical plates.
[e] Peak symmetry.

4.6. *Identification and quantification by HPLC–ICP-MS*

The percentage selenium distributions of different plant samples were determined using the PeakFit chromatographic software. The reported distribution numbers are relative percentage concentrations, where 100% is represented by the total selenium response of the eluting selenium compounds, including selenite and selenate. The sum of the identified selenium compounds is smaller than 100% in each case, with the unidentified compounds accounting for the missing portion.

The selenium species in *B. juncea* were identified from retention time comparisons with standards. The HPLC–ICP-MS chromatograms for the enzymatic extraction of selenate treated and selenite treated *B. juncea* shoots are shown in Figs. 3A and 4A, respectively. The major selenium species in the selenate treated samples is inorganic selenium (selenate) that comprised 82.8% of eluted selenium. The other identified selenium compounds were Se-methylselenocysteine (3.1%), selenomethionine (10.7%) and S-(methylseleno)cysteine (1.4%). The presence of the S-(methylseleno)cysteine in the *B. juncea* shoots, as also recently reported in nutritional yeast [33], is particularly noteworthy. An earlier study on wild type *B. juncea* treated with selenate indicated the presence of inorganic Se, Se-methylselenocysteine, selenomethionine, Se-homocystine and some unknowns in the *B. juncea* leaves [7].

For the plants treated with selenite, selenomethionine Se-oxide hydrate (51.2%) and selenomethionine (34.2%) were identified as the major organoselenium species. Selenite was present at a low level (4.3%). The presence of the high level of selenomethionine Se-oxide hydrate is noteworthy, considering its absence in the selenate treated plants. Substantially different redox conditions clearly apply in these two cases. In the study noted above for wild type *B. juncea* treated with selenite, the presence of inorganic selenium, selenomethionine, Se-methylselenocysteine and several unidentified selenium species in the leaves was also reported [7].

In the present study of *B. juncea* roots (Figs. 3C and 4B), in addition to inorganic selenium, selenomethionine was present at 27.8 and 43.1% in selenate treated roots and selenite treated roots, respectively. Recently Se-methylselenomethionine was identified and characterized in *B. juncea* roots when grown in the presence of selenomethionine [8]. Table 3 shows quantification of selenium species in plant shoots and roots for replicate analyses by HPLC–ICP-MS.

In the selenite enriched plant shoots, the concentration of selenomethionine (taken together with its oxide-hydrate) was higher than the concentration of inorganic selenium (selenite), most of the inorganic selenium being transformed to organoselenium species. The selenomethionine concentration in the selenite-enriched plants was higher in the plant roots (113 $\mu g\,g^{-1}$) than in the above-ground biomass (11 $\mu g\,g^{-1}$). These findings agree well with other reports

Table 3
Selenium distribution (%) in *B. juncea* plant shoots and roots by HPLC–ICP-MS

Selenium species	Se(IV)		Se(VI)	
	Shoots	Roots	Shoots	Roots
Selenate	–	0.9	82.8	59.6
Selenite	4.3	37.1	–	5.9
Selenomethionine Se-oxide hydrate	51.2	5.5	–	–
Se-methylselenocysteine	–	–	3.1	–
S-(methylseleno)cysteine	1.2	1.3	1.4	1.8
Selenomethionine	34.2	43.1	10.7	27.8

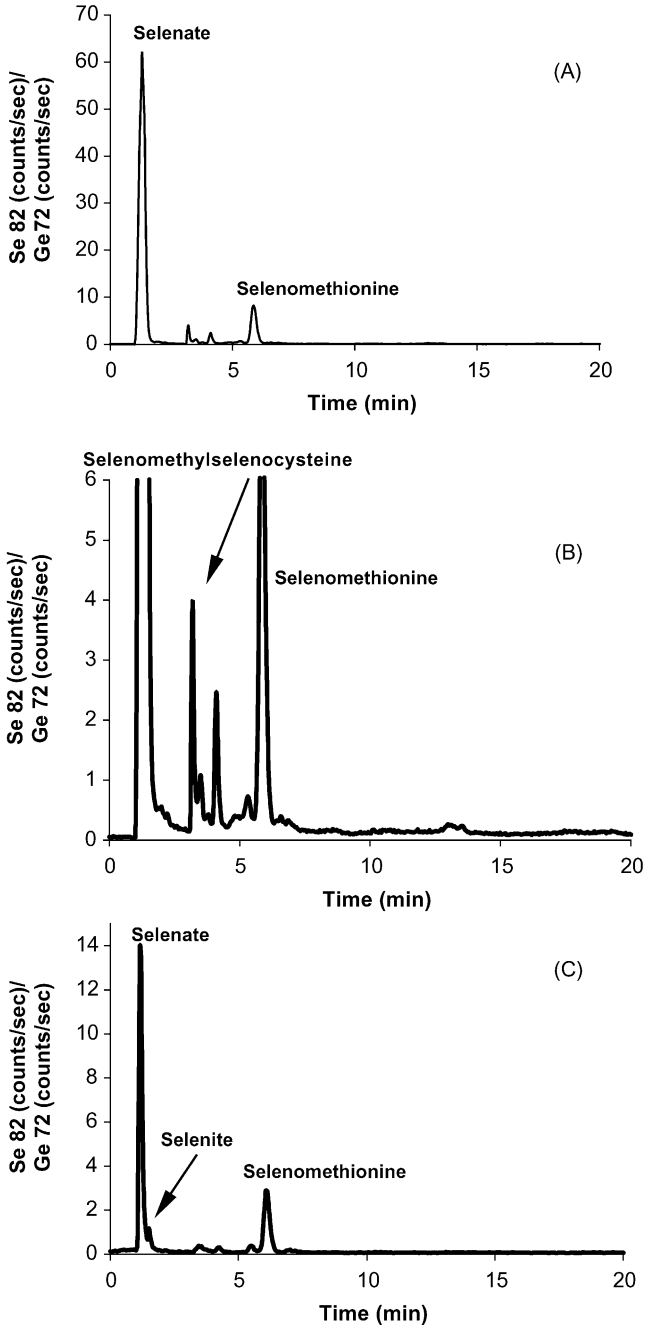

Fig. 3. HPLC–ICP-MS chromatogram of the enzymatic extraction of selenate (Se(VI)) treated *B. juncea* using 0.1% HFBA ion pairing agent, XTerra RP-C18 column. (A) *B. juncea* shoot extract (full scale); (B) *B. juncea* shoot extract (zoom); (C) *B. juncea* root extract.

that also indicate inorganic selenium is mainly metabolized in the plant roots, especially when selenite is added to the medium [34].

Some authors have suggested the use of selenium-enriched plants as a dietary product, especially material with selenomethionine and selenocysteine that may have antitumorigenic activity [15,16,35]. The chromatograms of a commercially available dietary supplement prepared from Indian mustard (*B. juncea*) and a selenized yeast sample are

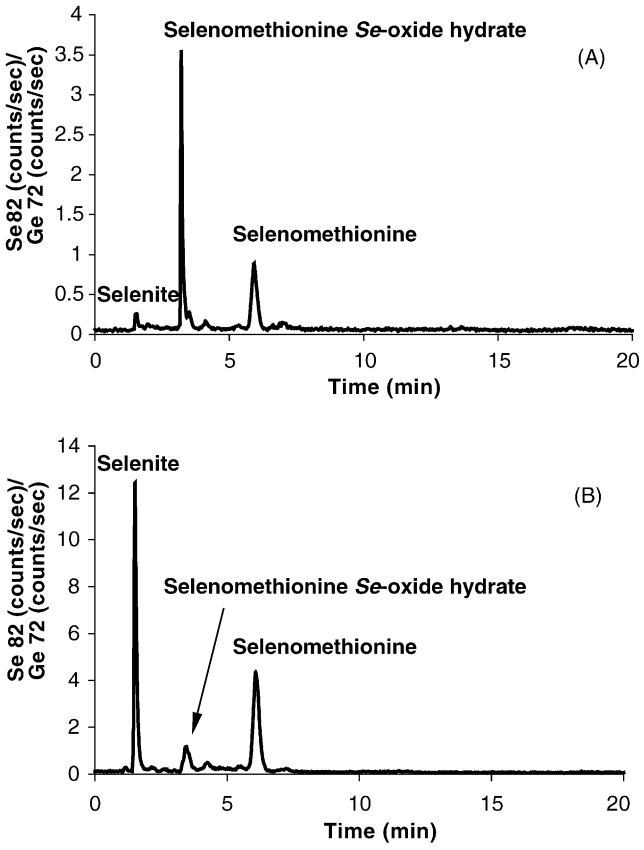

Fig. 4. HPLC–ICP-MS chromatogram of the enzymatic extraction of selenite (Se(IV)) treated *B. juncea* using 0.1% HFBA ion pairing agent, XTerra RP-C18 column. (A) *B. juncea* shoot extract; (B) *B. juncea* root extract.

shown in Fig. 5. The dietary supplement contains primarily inorganic selenium (selenate), suggesting no transformation of inorganic selenium to selenomethionine. According to our studies, this can be explained for example if the plants were grown in selenate enriched medium, where the selenium remains mainly as selenate in the plant shoots.

4.7. GC–AED of derivatized compounds

Extracts from the samples examined by HPLC–ICP-MS were also derivatized by ethylchloroformate to produce volatile ethylated species, which were determined by GC–AED. Fig. 6a is a selenium specific profile (Se at 196 nm) of the ethylchloroformate derivatives of six selenoamino acids, all of which elute within a 7 min window with excellent resolution and peak efficiencies. Fig. 6b illustrates the sulfur specific profile (S at 181 nm) of the ethylchloroformate derivatives of three sulfur containing amino acids. The retention times of these species are shown in Table 4. In general, the retention times of selenoamino acids are greater than those of analogous sulfur containing amino acids.

Fig. 7 shows comparative chromatograms of the shoots of the *B. juncea* plant treated with selenite, the selenized yeast sample (Fig. 5) and S-(methylseleno)cysteine reference stan-

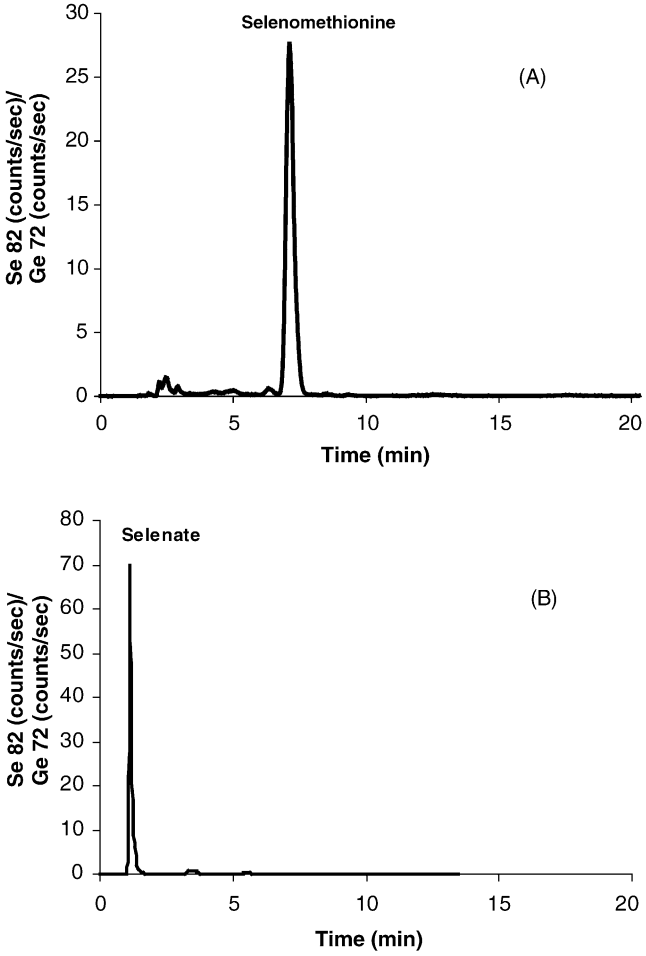

Fig. 5. HPLC–ICP-MS chromatograms of the enzymatic extraction of (A) selenized yeast and (B) *B. juncea* dietary supplement (200 μg Se) using 0.1% HFBA ion pairing agent, XTerra RP-C18 column.

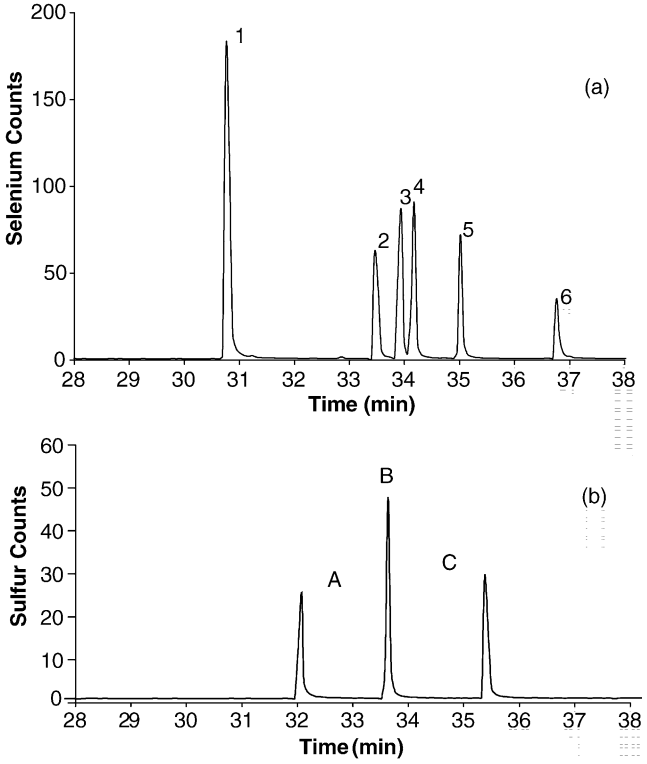

Fig. 6. GC–AED temperature programmed chromatograms, (a) derivatized selenoamino acids: (1) Se-methylselenocysteine derivative, (2) selenomethionine derivative, (3) Se-allylselenocysteine derivative, (4) Se-propylselenocysteine derivative, (5) selenoethionine derivative, (6) selenocysteine derivative; (b) derivatized sulfur-amino acids: (A) methionine derivative, (B) ethionine derivative, (C) cysteine derivative.

dards for comparison. Fig. 8 shows the chromatogram for the shoots of a *B. juncea* plant treated with selenate. The results from shoots and roots of plants treated with selenite and shoots treated with selenate are shown in Table 5. Inorganic selenium species are not shown, since they are not derivatized by ethylchloroformate. Thus, the relative percent data presented is not related to selenite or selenate that were shown by HPLC–ICP-MS measurements to comprise a major proportion of selenium speciation, particularly for selenate treated shoots. Data for selenate treated roots was not obtainable by GC–AED since the sample appeared to degrade on storage prior to analysis. Further data will be presented subsequently.

Selenomethionine was the major species present in the shoots of plants treated with selenite and selenate as shown in Figs. 3A and 4A. S-(methylseleno)cysteine [30] was also confirmed in the shoots of the plants treated with selenite and selenate. The values for the ratio of selenomethionine to S-(methylseleno)cysteine in Table 5 bear reasonable agreement to those seen in Table 3. Absolute values for individual

Table 4
Ethylated selenium- and sulfur-amino acid standards molecular weights (Se 80 and S 32) and retention times

Ethylated standard	Molecular weight	GC retention time (min)
Se-methylselenocysteine derivative	283	30.75
Selenomethionine derivative	297	33.52
S-(methylseleno)cysteine derivative	315	35.50
Se-allylselenocysteine derivative	309	33.95
Se-propylselenocysteine derivative	311	34.20
Selenoethionine derivative	311	35.00
Selenocysteine derivative	341	36.75
Methionine derivative	249	32.07
Ethionine derivative	263	33.64
Cysteine derivative	293	35.38

Table 5
Selenium distribution (%) in *B. juncea* plant shoots and roots by GC–AED

Selenium species	Se(IV)		Se(VI)
	Shoots	Roots	Shoots
Se-methylselenocysteine	N.D.	N.D.	–[a]
Selenomethionine	85.5	76.4	48
S-(methylseleno)cysteine	6.3	7.1	3.1

N.D., not detected.
[a] Below limit for quantitation.

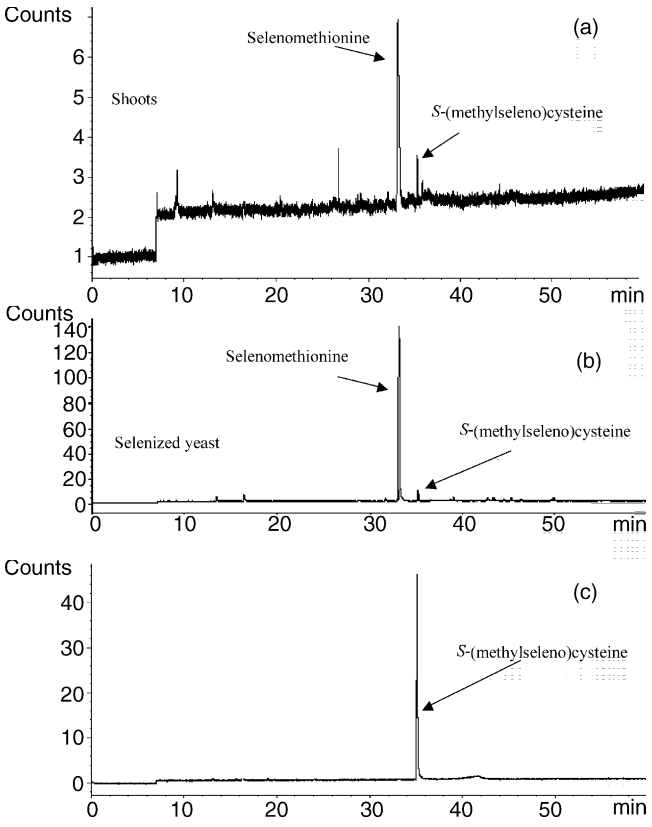

Fig. 7. GC–AED (Se 196 nm) of ethylated derivatives of (a) selenite treated shoot extracts; (b) selenized yeast; and (c) S-(methylseleno)cysteine standard.

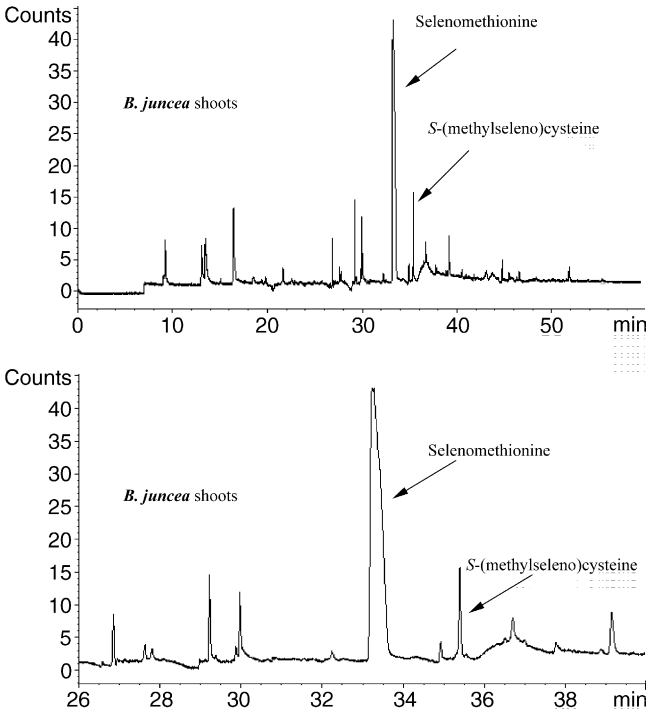

Fig. 8. GC–AED (Se 196 nm) of ethylated derivatives of selenate treated shoot extracts (top), and expanded scale 26–40 min (bottom).

compounds are different because of the absence of inorganic Se speciation by GC–AED.

5. Conclusions

In selenate treated *B. juncea* plants Se-methylselenocysteine, selenomethionine and S-(methylseleno) cysteine were identified by HPLC–ICP-MS and GC–AED. In selenite treated *B. juncea* plants only selenomethionine and S-(methylseleno)cysteine were speciated using both element specific detection techniques. Differences in selenium uptake and species found in the same plant type could also result due to different environmental conditions during selenium accumulation. There were several advantages of using HPLC–ICP-MS and GC–AED for selenium speciation in plants. HPLC–ICP-MS provided information about inorganic selenium species and the presence of selenomethionine Se-oxide hydrate. A major advantage of GC–AED is the ability to display simultaneously a number of element specific chromatograms for different elements such as, carbon, sulfur and selenium. In this fashion Se–S containing species can be identified in order to help elucidate the selenium metabolic pathway in selenium accumulators. The current study demonstrates that selenium can accumulate at different levels in *B. juncea* shoots and roots for different treatments. Nutrition solutions containing up to $5\,\mu g\,mL^{-1}$ selenium as selenite or selenate did not have an adverse effect on plant growth during this study. Studies on selenium accumulation may compliment enrichment efforts for phytoremediation studies and result in superior selenium sources in human diets.

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